Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/017829

International filing date: 07 June 2004 (07.06.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/476,664
Filing date: 06 June 2003 (06.06.2003)

Date of receipt at the International Bureau: 16 August 2004 (16.08.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

1204093

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

August 08, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: *60/476,664*
FILING DATE: *June 06, 2003*
RELATED PCT APPLICATION NUMBER: *PCT/US04/17829*

Certified by

Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office

6-9-03 PTO/SB/16(10-01)

Approved for used through 10/31/2002. OMB 0651-0032

Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

PROV

14230 U.S. PTO

16447 U.S. PTO 60/476664 06/06/03

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

TO: Mail Stop Provisional Patent Application
Commissioner for Patents
U.S. Patent & Trademark Office
P.O. Box 1450
Alexandria, VA 22313-1450

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

Atty. Docket No.	UOM 0300 PRV

INVENTOR(s)

FIRST NAME & MIDDLE INITIAL	LAST NAME	RESIDENCE (CITY & EITHER STATE OR FOREIGN COUNTRY)
Shuichi	Takayama	3550 Dixboro Lane, Ann Arbor, MI 48105
Gary D.	Smith	1325 Olivia Ave., Ann Arbor, MI 48104
Ronald	Suh	2012 Runnymede Blvd. Ann Arbor, MI 48103

____ Additional inventors are being named on the ___ separately numbered sheets attached hereto.

TITLE OF THE INVENTION *(500 characters max.)*

INTEGRATED MICROFLUIDIC SPERM ISOLATION AND INSEMINATION DEVICE

DIRECT ALL CORRESPONDENCE TO WILLIAM G. CONGER:

22045
PATENT & TRADEMARK OFFICE

ENCLOSED APPLICATION PARTS *(check all that apply)*

X Specification - Number of Pages 38 ____ CD(s), Number ____
X Drawing(s) - Number of Sheets 8 ____ Other: Specify ____
____ Application data sheet. See 37 CFR 1.76.

METHOD OF PAYMENT OF FILING FEES *(check one)*

	PROVISIONAL FILING FEE AMOUNT(S)	AMOUNT SUBMITTED OR TO BE CHARGED TO DEPOSIT ACCT.
X Applicant claims small entity status. See 37 CFR 1.27. X A check or money order is enclosed to cover the Provisional Filing fees. ____ The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number:	\$160.00 (large) \$ 80.00 (small)	\$ 80.00

X Please charge any additional fee or credit any overpayment in connection with this filing to our Deposit Account No. 02-3978.

X The invention was not made by an agency of the United States Government or under a contract with an agency of the United States Government.

____ The invention was made by an agency of the U.S. Government or under a contract with an agency of the U.S. Government. The name of the agency is ________. and the contract number is _______.

X A return postcard is enclosed.

Respectfully submitted,

SIGNATURE [signature]
REGISTRATION NO. 31,209

DATE June 6, 2003
TYPED or PRINTED NAME William G. Conger
TELEPHONE (248) 358-4400

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence is being deposited with the United States Postal Service via Express Mail Label No. EV 227 574 958 US in an envelope addressed to: Mail Stop Provisional Patent Application, Commissioner for Patents.U.S. Patent & Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450 on:

June 6, 2003 — LIDIA FREISMUTH — [signature]
(Date of Deposit) (Name of Person Signing) (Signature)

Integrated Microfluidic Sperm Isolation and Insemination

1) General Purpose: Experiments were performed to demonstrate proof-of-concept that a Microfluidic Integrated Sperm Sorter (MISS) could be used to isolate motile sperm from non-motile sperm and debris, that these isolated sperm could be used to inseminate oocytes in isolation wells or microfluidic channels, and the sperm binding and fertilization would ensue.

2) Technical Description: The MISS made of PDMS was used to isolate motile mouse sperm from non-motile sperm and debris by inter-streamline crossing. Sperm in media were loaded into a loading well (A); media was loaded into well (B). The gravity driven pumps in these loading wells provided flow. When channels from A and B come together they form laminar flow whereby only motile sperm can cross the inter-streamline between the two solutions. When the streamlines separate the fluid emptying into well (C) has nonmotile sperm and debris and the well (D) has motile sperm. Eggs can be placed into well D; motile sperm will bind to the egg's zona pellucida and fertilize the oocytes. PMSD is nontoxic to eggs and preimplantation developing embryos. In addition, eggs can be placed into a continual flow Mircofluidic Egg Insemination Device (MEID) made of PDMS, will stop their motion down the channel at a "Catch Site" while media continues to flow past the egg. Sperm from the MISS can be introduced into the MEID, will flow to the egg, bind to the zona pellucida and fertilize the egg.

3) Advantages/Improvements Over Existing Methods/Devices: Motile sperm can be isolated from nonmotile sperm and debris without centrifugation. Motile sperm can be isolated from seminal plasma with out centrifugation of mixing of seminal plasma and media. Very few motile sperm can be separated from nonmotile sperm and debris. The very few motile sperm isolated with the MISS can be used to inseminate and fertilize the egg with no sperm manipulation. Integration of the MISS with the MEID, where MEID channels are not much larger then the egg itself, will allow insemination with very few motile sperm. The few motile sperm that travel in the MEID flow will have to come into contact with the eggs at the Catch Site, resulting in micro-insemination at a level that can not be accomplished with current human/animal in vitro fertilization techniques. This integration of the MISS and MEID may replace the need for intracytoplasmic sperm injection (ICSI) in many human assisted reproductive technologies. Integration of the MISS and MEID will allow domestic farm animal single ejaculates or frozen samples to be used to inseminate more eggs from more females. Integration of the MISS and MEID may provide means of propagating genetically altered rodent species that currently is quite difficult (ie. use of frozen sperm from transgenic males-very poor cryo-survival = few motile viable sperm; to inseminate many eggs).

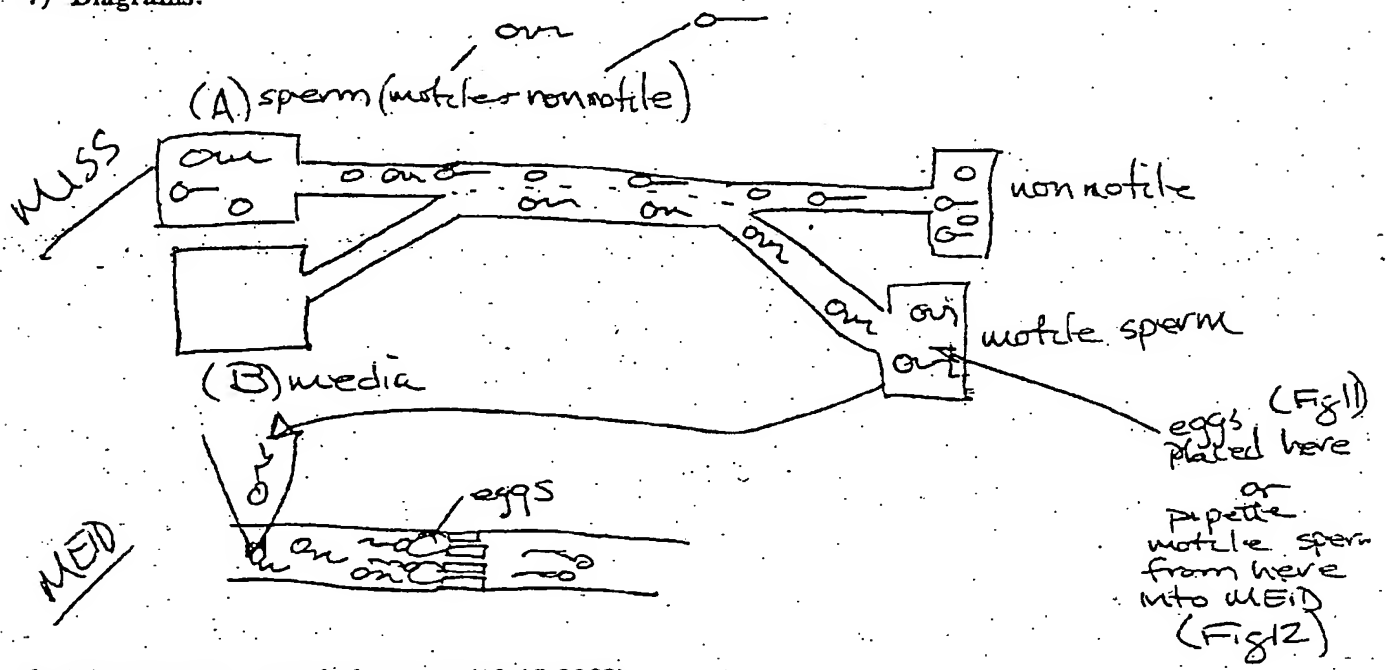
4) Possible variations/modifications: Build a microfluidic device whereby the sorted motile sperm instead of entering a well (D) go straight into a micro-channel in which eggs are placed for insemination.

5) New Features: Microfluidic insemination and fertilization of eggs. Use of gravity-driven microfluidic pumps to isolated motile sperm for insemination. Use of motile sperm isolated

with the MISS to inseminate and fertilize eggs. Integration of the MISS with an insemination device. Use of continual media flow to introduce sperm to eggs at a catch site whereby all sperm introduced into the microfluidic channel must come into contact with eggs.

6) **Related Patents:** None

7) **Diagrams:**



8) **Micrographs** - proof of concept (12-17-2002)

The MEID may be separate from the MISS, but is preferably integral therewith. The chambers of the MEID which contain the eggs for fertilization may be configured of any convenient size. However, as indicated, the chambers are preferably not appreciably larger than the egg itself, for example, but not by limitation, in the range of 1.1 times the egg diameter to 10 times the egg diameter, preferably 1.2 times the egg diameter to about 2 times the egg diameter. The length of the chamber may be such as to be able to accommodate but a single egg, or a plurality of eggs, preferably "in series", such that sperm which bypasses a first egg may contact a second or further egg.

The eggs may be plated into the device by customary techniques, or may be "trapped" by a constriction in the egg chamber, or be a post, a series of parallel channels with walls in between, etc. The devices may be made of numerous materials, as for the gravity driven pumps and sperm sorters, but are preferably constructed of silicone materials.

Microfluidic devices are steadily supplanting their macroscale counterparts in numerous applications, particularly in biological and pharmaceutical research. Such devices often require one or more pumps to propel fluid through microchannels. Current methods may employ mechanical pumps such as syringe-type pumps and micromechanical pumps, and non-mechanical pumps such as electrohydrodynamic pumps, electro-osmotic flow pumps, electrowetting pumps, and thermocapillary pumps.

All these pumping systems have drawbacks associated with them. For example, a steady flow rate is difficult to achieve. Moreover, mechanical pumps require an electrical power source, as do pumps which operate based on electrical properties. Most of these pumps are costly and inconvenient to integrate with other microscale devices, and often have slow response times. Electro-osmotic flow pumps have a flow profile which is pH dependent, and which produce stable flow only over a limited pH range. Recently, a thermocapillary pump has been developed. However, this pump requires complex electronic control circuitry and creates heat transfer issues.

The use of gravity-driven flow in microfluidic applications has been attempted. However, conventional reservoirs produce a decrease in hydrostatic pressure as the liquid level in the reservoir drops. This decreasing pressure difference leads to decreased flow rates with respect to time.

It would be desirable to provide a microfluidic system including a microfluidic pump which is simple, economical to produce, and preferably disposable, which can provide a relatively constant and optionally adjustable flow rate, without the necessity for an external power source.

It has now been surprisingly discovered that a gravity driven microfluidic pump comprising a substantially horizontal supply reservoir can provide a substantially consistent flow rate in microfluidic devices which does not change as the pumped volume increases over time. The pump system of the present invention has numerous applications in microfluidic devices, including applications in *in vitro* testing of pharmaceutical products in environments which mimic those *in vivo*, in cell and embryo culture, cell sorting and other applications.

FIGURE 1 illustrates schematically one embodiment of a microfluidic device employing a gravity driven pump of the present invention;

FIGURE 2 illustrates flow rate against time of an embodiment of Example 1 of the present invention;

FIGURE 3 illustrates flow rates at various heights of the device of Example 1;

FIGURE 4 illustrates the linear relationship between height and flow rate of the device of Example 1;

FIGURE 5 illustrates a microfluidic device having converging inlets and the flow produced therein in accordance with Example 2;

FIGURE 6 illustrates the effect of concentration of BSA an flow rates in the device of Example 1;

FIGURE 7 illustrates the effect of temperature on flow rate in accordance with Example 4;

FIGURE 8 illustrates a microfluidic device useable with the subject invention gravity driven pump to monitor embryo development.

FIGURE 9 illustrates one embodiment of an integrated gravity driven pump, microchannel, and outlet reservoir; and

FIGURE 10 illustrates a further embodiment of an integrated gravity driven pump, microchannel, and outlet reservoir.

The subject invention gravity driven microfluidic pump system comprises a substantially horizontally-oriented fluid reservoir connectable to or connected to a microfluidics device having at least one microchannel through which supply liquid flows. In preferred devices, a horizontally oriented outlet reservoir receives fluid from the microchannel(s).

The fluid supply reservoir is substantially horizontal, and is of sufficient size to carry the desired amount of fluid for sustained operation. By the term "substantially horizontal" is meant a horizontal or nearly horizontal position such that the hydrostatic pressure asserted by the liquid in the supply reservoir is relatively constant. A horizontal reservoir may deviate from the absolute horizontal orientation to generate a pumping system where the hydrostatic pressure is intentionally caused to vary over time. In general, an orientation which deviates from horizontal by about 10° or less, preferably 5° or less is suitable. A very slight incline toward the outlet of the supply, i.e. 1-3°, may be useful to counter effects due to surface tension between the liquid being pumped and the walls of the reservoir. Moreover, in actual pump configurations in the laboratory, it may be difficult to adjust the orientation of the device to a purely horizontal configuration.

Due to the volume generally required to supply fluid over time, the volume of the supply reservoir is normally considerably greater than the volume of the microchannel(s) through which fluid flow is desired. For example, at flow rates of 1000-3000 nL/hr, the reservoir may be several mL in volume. The reservoir volume is generally at least 10 times the volume of the microchannel(s), preferably more than 100 times the volume of the channel. The reservoir preferably has an aspect ratio (length: inside diameter) of at least 5:1, preferably at least 10:1, and more preferably at least 100:1.

The reservoir may constitute a straight run of hollow tubing, for example of glass, and may also be bent in a U-configuration or in a horizontal spiral to conserve space. A suitable configuration is shown in Figure 1, wherein fluid in supply reservoir 1 flows through tubing 2 to microfluidics device 3, in this case having a single microchannel 4. From microchannel 4, fluid flows through tubing 5 to outlet reservoir 6, the "shadowed" ellipses indicating the positions of the menisci in the supply and outlet reservoirs after a period of flow has occurred. The internal cross-section of the hollow tubing may be of any desired shape. Preferably, the internal cross-section is round, but elliptical, square, rectangular, octagonal, ("polyhedral") or other cross-sections may be used as well. The cross-sectional shape of the reservoir may be of any shape (circle, square, rectangle, etc) as long as the shape is such that it maintains enough surface tension to keep the fluid inside from spilling out. The cross-sectional shape may vary along the length of the reservoir to program different hydrostatic pressures at different points in time determined by the amount of fluid volumes present in the reservoirs. The supply reservoir may be a separate device connected permanently to, or connectable to, the microfluidic device, or may be integrated into the device. For example, a glass tubing reservoir may have standard microfluidics connectors which enable connection to a microfluidic device channel through suitable tubing, for example hollow tubing of polydimethylsiloxane elastomer ("PDMS"), polysulfone, etc. Alternatively, a PDMS or other polymer device may be fabricated to contain both the supply reservoir and the microchannel through which flow is desired as shown in but two embodiments in Figures 9 and 10. In such devices, at least a portion of the supply reservoir and the microchannel(s) are at different heights, so that when

positioned such that the supply reservoir is horizontal, a gravity-created hydrostatic pressure condition exists between the reservoir and the microchannel outlet. The microchannel may be horizontal, inclined or vertical, preferably horizontal.

The diameter of the fluid supply reservoir is only critical to the extent that the surface tension between the fluid and the walls of the reservoir is sufficient to maintain the liquid within the reservoir at a given reservoir internal diameter, and the internal diameter is not so small as to prevent fluid flow due to the surface tension. This relationship between surface tension and internal diameter will vary depending upon several factors, including the geometry of the internal cross-section of the reservoir, the nature of the inner walls of the reservoir, and the nature of the fluid. The suitability of any particular reservoir may be assessed easily by filling the reservoir with liquid and observing whether the reservoir will hold the liquid stably through the microchannel when connected thereto.

For example, reservoirs of square or rectangular cross-section will be expected to allow for larger "diameters" than those of circular cross-section due to increased interaction between the fluid and the walls at the corners of the internal cross-section and the higher surface to volume ratio of non-circular cross-sections. For aqueous fluids, internal walls which are hydrophilic will exhibit lesser fluid/wall interactions than surfaces which are less hydrophilic (or more hydrophobic). Finally, the nature of the fluid itself is important. The presence of surface tension-lowering compounds in the fluid will alter the maximum "diameter" accordingly.

For example, with a fluid which contains 1.0 weight percent BSA (bovine serum albumin), glass tubing of diameters from 2.5 mm to 6.0 mm were tested. It was found that with this fluid, 5-6 mm inside diameter ("I.D.") tubing was incapable of holding fluid by surface tension, whereas tubing of 2.5 mm exerted too high a surface tension, preventing fluid flow or minimizing fluid flow to such an extent that the pump is essentially inoperable. However, tubing with nominal diameters between 3.5 mm and 4.0 mm were found to be eminently well suited as a reservoir for such applications. The suitability for any given internal "diameter"/configuration can be simply assessed by ascertaining, first, whether fluid

can be held within the reservoir by orienting the reservoir horizontally and determining that the fluid will not flow out of its own accord; and second, by ascertaining that the interaction between the wall of the reservoir and the fluid is not so high that the desired flow rate between the reservoir and a given microchannel cannot be obtained.

The microfluidic device contains at least one microchannel through which fluid flows due to hydrostatic pressure exerted by a difference in height (relative to gravitational field) between the supply reservoir and the microchannel. The microfluidic device may contain but a single microchannel, may contain a plurality of converging microchannels, may contain a plurality of parallel microchannels, or any combination thereof. The microchannels must be of sufficient size such that flow of fluid is possible. In other words, the internal diameter of the microchannels must not be so small that the devices "lock up" once fluid full, whereby fluid flow is prevented. In general, the diameter of the microchannels, if circular, is from 1 μm to about 5 mm, preferably 10 μm to about 3 mm, more preferably 100 μm to 2 mm. As with the supply reservoirs, the internal size is related to the channel cross-section, hydrophilicity/hydrophobicity, and fluid nature.

The microchannels of the device are terminated in an outlet. The outlet is preferably in fluid communication with a fluid reservoir, in order to prevent fluctuations in pressure associated with formation of "drops" from the outlet. The outlet reservoir may be a simple container with which the microchannel communicates, or preferably is an outlet reservoir of the same size, material, and geometry as the supply reservoir. In such a situation, capillary forces cancel out and hydrostatic pressure due to gravity is the only driving force for the liquid.

When the supply reservoir and outlet reservoir are of the same configuration and construction, the effects of geometry-induced and constitution-induced (i.e. hydrophilic/hydrophobic) properties may be completely offset, thus increasing the constancy of flow. For example, when the supply reservoir consists of a 4 mm I.D. pyrex glass tube, the outlet reservoir may also consist of a 4 mm

I.D. pyrex glass tube as well. In such cases, once the device is initially filled with fluid, i.e. by application of a slight pressure differential between the supply reservoir and the outlet, fluid flow may occur from reservoirs whose internal size is too small for flow without the use of a similar outlet reservoir.

The effects of surface tension in the supply and outlet reservoirs may be changed by rendering these surfaces increasingly hydrophilic or hydrophobic, or by surface treatments which are specific to fluid components. For example, glass surfaces may be rendered more hydrophobic by reaction of the surfaces with hydrophobicizing compounds such as octyltrimethoxysilane and perfluoropropyltriethanoxysilane and like compounds. Such silanes react with silanol groups on the silica surface. If a glass surface is desired to be rendered more hydrophilic, it may similarly be reacted with silanes which bear hydrophilic groups, i.e. polyoxyethylene/polyoxypropylene groups or glycosidyl groups. For other than silica-based reservoirs, for example of organic polymers, numerous reagents are known which effect hydrophilization/hydrophobicization. Use of perfluoroalkyl compounds allows alteration of surface tension both with respect to water (aqueous compositions) as well as oleaginous compositions (i.e. paraffinic solvents). A net capillary force may also be generated by the reservoirs by adjusting the cross-sectional areas of the reservoirs. In these cases there would be a combination of gravitational and capillary force that would pump the fluid.

The fluid flow of gravity driven pumps of the subject invention is easily adjusted. For example, positioning the height of the supply reservoir at increasing heights above the outlet reservoir can be used to increase fluid flow appropriately. If fluid flow is desired to be altered during operation of the device, a robotic device may be manually or programmably directed to increase or decrease the relative height differences between the inlet and outlet. Thus, the flow rate can be increased or decreased in any manner, i.e. linearly, sinusoidally, stepwise, etc.

Programmed flow rates may also be provided without resort to robotic devices or the like by altering the nature of the walls of the supply or outlet reservoirs at specific locations, or their internal size. For example, in a cylindrical

supply reservoir such as a length of glass tubing, a first portion of the internal walls of the tubing may be rendered hydrophobic while a second portion may be hydrophilic. The rate of flow will alter in a stepwise manner when the liquid in the tube reaches the boundary between the hydrophobic and hydrophilic surfaces due to a change in surface tension at this point. Alternatively, an inlet reservoir may be configured to have succeeding portions stepped in height, for example a portion most remote from the microchannel(s) to which fluid is supplied having a height higher than a closer portion. The flow will be constant from the first (higher) portion of the reservoir, and then fall in a step fashion as the fluid level in the higher reservoir falls to the level in the lower reservoir, when flow will again remain constant, but at a lower rate.

The channels of the microfluidic devices may be treated to encourage adhesion of a variety of substrates whose interaction with the supply fluid and/or its components is desired. The treatment of the microchannels may take place prior to assembly of a complete microfluidics device, or may take place after assembly. In preferred devices, treatment of the microchannel surfaces takes place prior to assembly of the device.

The microfluidic devices used in accordance with the subject invention may be any microfluidic device, particularly microfluidic devices for which a relatively constant fluid flow is desired. Numerous uses are possible, including motile sperm sorters, B. Cho, et al. Proceedings of the IEEE-EMBS Conference on Microtechnologies in Medicine and Biology, pp. 156-159 (2002), and cell size sorting, D. Huh, et al., Proceedings of the 2nd IEEG-EMBS Conference, pp. 466-469 (2002), incorporated herein by reference.

For example, in the *in vitro* testing of pharmacologically active compounds, it is recognized that a difference in observed *in vitro* activity is observed as opposed to *in vivo* activity. This discrepancy is believed by some to be the result of exposure of cells and cell components to presence of fluid flow *in vivo* whereas most assessments of activity *in vitro* are assessed under static conditions. For example, mechanical extracellular forces, in particular on endothelical cells and

smooth muscle cells, have been studied extensively. Laminar sheer stress within blood vessels cause endothelial cells to express factors which inhibit platelet coagulation, leukocyte adhesion and migration, LDL accumulation, and endothelial cell maintenance. Mass transport also changes with fluid flow. Delivery of oxygen, nutrients and removal of waste materials and autocrine factors are also affected by fluid flow. An effective tool to monitor these flow-related phenomena under steady flow rather than under static conditions is needed; the present invention supplies the ability to do so in a cost-effective and efficient manner.

Thus, the present invention further pertains to a method of observing and/or analyzing flow related sheer stress-related phenomena in biological systems where the analysis requires or prefers a steady flow of fluid as is provided by the gravity driven microfluidics pumps of the present invention.

Within microfluidic culture environments for human and non-human embryos, a dynamic culture system holds numerous advantages in comparison to current static culture systems. First, gradual movement of media over embryos would have the ability to remove metabolic by-products such as ammonia and oxygen free radicals which are detrimental to embryo development. In addition, individual blastomeres (cells) that comprise the embryo can undergo apoptotic death, fragmentation and release of apoptotic cell death agents that may be detrimental to survival of remaining blastomeres. A dynamic culture system would remove such agents. Second, current human embryo culture strategies use 2-3 sequential media for 3-6 day culture with abrupt media changes, which can inflict osmotic stress upon embryos. A dynamic culture system would allow gradual media changes that may be beneficial. Third, within the oviduct, cilia of epithelial cells are continuously "beating" causing constant movement of preimplantation embryo(s). Such movement, which can be achieved with dynamic media flow, may be beneficial for establishing poles-of-cell division and enhance embryo developmental competence. Fourth, dynamic media flow over embryos will allow "sampling" of embryo by-products that have potential of indicating which embryos have the greatest change of implantation and pregnancy establishment. Lastly, group embryo culture is believed to be superior to individual culture based on the idea that more

developmentally advanced embryos ("helpers") produce substances that enhance poorer embryo ("lagger") development. Conversely, poorer developing embryos may have detrimental influence on more advanced embryos. Culture devices using dynamic media flow may facilitate "helper" embryo influences on "lagger" embryo development without negative reciprocal effects. For the effects of fluid flow on embryo development, reference may be had to S. Nonaka et al., "Determination of Left-Right Patterning of the Mouse Embryo by Artificial Nodal Flow," NATURE, 418, pp. 96-99, July, 2002.

A device suitable for studying embryo development is illustrated in Figure 8. In Figure 8, the microchannel 10 has a portion 12 which is constricted in at least one dimension or bears a grid, post, or other structure capable of holding an embryo in place under flow conditions. The embryo may be introduced into the microchannel by numerous means, as shown in Figure 8 by a converging channel 13 which is sealed off once the embryo is in place. Other means which restrict the clear path, such as a grid, post, protuberance, etc., are also acceptable, so long as flow around the embryo can be maintained. The upstream end 14 of the microchannel is connected to horizontal supply reservoir 15 by tubing or passageway 16 which the downstream end 17 is an outlet, preferably connected to outlet reservoir 18 by tubing or passageway 19.

The rate of flow produced by the pumps of the present invention is adjusted by altering the height of the supply reservoir above the microfluidics device. For example, heights from 0.1 mm to about 4 m are suitable, more preferably from 1 mm to 1 m, yet more preferably from 10 mm to 750 mm, and most preferably in the range of 10 mm to 100 mm. Devices with low flow rates and concomitantly low supply reservoir heights are suitable for single device fabrication.

Among preferred embodiments of the subject invention, microfluidic devices are those wherein a supply reservoir and an outlet reservoir are constructed integrally with the microfluidic channel(s), and those wherein the device is constructed such that the microchannel can temporarily retain an open side onto which cells can be plated prior to sealing off the microchannel.

In the first of these preferred embodiments, supply and reservoir channels may be fabricated on the same substrate as the microchannel itself. Such devices then need only to be supplied with the appropriate fluid(s) to function. The reservoirs and microchannels of two such devices are shown in Figures 9 and 10. In both these devices, the supply reservoir is considerably greater in volume than the microchannel volume so that flow may occur for some length of time. In Figure 9, the supply reservoir 91 is somewhat larger in area and taller than the outlet reservoir 93. When filled with fluid, the height difference of the two reservoirs allows fluid flow through microchannel 92 until the heights of fluid in the two reservoirs is equal. In Figure 10, the outlet reservoir 96 is configured lower than the supply reservoir 94 in its entirety, allowing complete emptying of the supply reservoir through microchannel 95 providing the volume of the outlet reservoir is sufficient to accept the required fluid volume.

In the second of the above embodiments, the device may be cast, for example of PDMS, and upon removal from the mold, the microchannel and reservoirs, when present, will be open on the side facing the mold. These surfaces can be appropriately plated with cells, or treated with substances which promote or inhibit cell adhesion. The various channels and/or reservoirs may also be closed temporarily to facilitate single or multiple coating/plating processes. For example, portions of the device, for example, the channel may be coated with cell adhesive proteins in the closed channel state, and other surfaces coated with non-cell adhesive protein. The device may be peeled of its temporary "closing" backing, and then, in the open state, cells may be plated. Plating cells in the open state, before making the closed channel system, while achieving selective cell attachment inside the channel, is important for allowing rapid plating of cells into the channels. After cells attach, the channel system is closed again.

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Example 1

A microfluidic device is constructed from PDMS by micromachining a silicon substrate and casting a curable PDMS onto the substrate to obtain a PDMS slab with embedded channel features. Reference may be had to S. Takayama, et al. "Patterning Cells and Their Environment Using Multiple Laminar Fluid Flows in Capillary Networks," PROC. NATL. ACAD. SCI. USA, pp. 5545-48, 1999; Duffy et al. "Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane), ANAL. CHEM. 70, pp. 4974-84, 1998. The microchannel is ca. 4 mm in width. The channel is sealed by conformal contact with a planar PDMS slab.

A 5/32" (4 mm) inside diameter glass tube is employed as the supply reservoir and is positioned horizontally on a stand with one end open and the other (supply) end connected with a 0.030 (0.75 mm) I.D., 0.065 O.D. (1.65 mm) silicone tubing (VWR Scientific). The silicon tubing is sealed to the inlet of the microchannel of the PDMS microfluidic device with epoxy resin. The outlet of the microchannel is similarly sealed to another piece of silicone tubing, which is connected to a glass tube (outlet reservoir) of the same diameter as the supply reservoir.

To initially fill the channel system with liquid, the liquid-filled reservoir is held high and optionally tilted to allow rapid loading of the microchannel. The supply reservoir is then situated approximately horizontally at a level similar to that of the outlet glass reservoir, the latter serving as the fluid outlet reservoir. The supply reservoir is then raised above the level of the outlet reservoir, and the meniscus at the front of the pumped liquid monitored using a horizontally oriented stereoscope (Nikon SMZ-1500). Time lapsed images are captured with a CCD camera (Hamamatsu Orca-100) to monitor the flow meniscus in the horizontally positioned flow supply reservoir at 60 sec. intervals. Resolution between adjacent images is about 80 pixels. The flow rate is plotted against time and found to be substantially constant, as indicated by Figure 2, a plot of flow rate in nL/min versus time, with a slope of $y=-0.0102 x +456.72$. Less than a 1%

variation in flow rate is demonstrated, within the limitation of the resolution of the CCD (80 pixels/meniscus front = 1.25%).

The flow rate at various supply reservoir heights relative to the outlet reservoir is assessed similarly. A plot of flow rates is shown in Figure 3, where 1 represents a height of 19.5 mm, 2 a height of 33.3 mm, 3 a height of 44.7 mm, 4 a height of 50.3 mm and 5 a height of 75.5 mm. The relationship between supply reservoir height and flow rates is substantially linear, as shown in Figure 4.

Example 2

In the same manner as Example 1, a multiple-inlet microfluidics device is fabricated from PDMS, having a central inlet and two flanking inlets. The central inlet is connected to a substantially horizontal fluid reservoir of water containing a red dye, whereas the flanking inlets are connected to a water reservoir. The flow is illustrated by Figure 5. The widths and shape of the flow remained unchanged for 36 hours. The microchannel width is about 300μm, and each of the supply channels is about 100 μm.

Example 3

The effect of different fluids on the flow rate is shown by Figure 6, wherein the flow rate at various heights are monitored with three different concentrations of BSA: 0.1% by weight, 1% by weight, and 10% by weight. As expected, the flow rate declines with increasing BSA concentration.

Example 4

The flow rate of 1% BSA is monitored at different temperatures. A plot of the temperature dependence is shown in Figure 7, the flow rate increasing from about 4000 nL/min. at ca. 14°C to ca. 6300 nL/min at ca. 27°C.

Example 5

The use of the present devices employing constant flow pumps for cell plating/culture is demonstrated by plating c2c12 cells onto select regions of a microfluidic device. It has proven difficult to provide high cell density in completely closed devices; hence, cell plating is accomplished prior to "closing" of the microchannel.

A negative substrate is produced onto which PDMS is cast, providing a 3 cm long microchannel having a height of 40 μm and width of 1 mm, an inlet reservoir 3 mm in height and 4 mm in width, and an outlet reservoir of 2 mm height and 4 mm width on either side of the microchannel.

The PDMS layer is removed from the "master," and the channel, along with a trough (to be filled with media and cells) are sterilized under UV light for 20 minutes, following which the PDMS slab with channel is placed on a glass slide with the channels facing the glass slide. The inlet reservoir is filled with 2% BSA solution, and incubated at room temperature for 30 mins. The BSA solution does not enter the microchannel due to its hydrophobicity and lack of hydrostatic pressure at the inlet reservoir. The BSA solution is sucked out of the inlet reservoir by a pipet resulting in a BSA coated inlet reservoir. The channel and outlet are not coated.

A cell adhesive protein, such as collagen DQ, is introduced into the outlet reservoir and forced into the channel, following which the device is incubated at room temperature for 45 minutes. BSA solution (2%) is again introduced into the inlet reservoir, and forced into the channel employing pressure at the inlet or suction at the outlet to rinse remaining collagen from the channel. The outlet reservoir is rinsed with 2% BSA solution as well.

The PDMS layer is peeled off the glass slide and covered with 2% BSA, followed by incubation at room temperature for 30 min. The BSA solution is rinsed off the device using sterile Phosphate Buffered Saline ("PBS"), and the PDMS layer is stored in a BSA-containing Petri dish (to prevent drying out). The

PDMS channel is then placed in a petri dish with channel opening facing up and exposed, and the device covered with media, consisting of Dulbecco's Modified Eagle Medium ("DMEM") plus 10% fetal bovine serum ("FBS") plus 1% Penicillin G.

A culture dish with cells was evaluated to ensure that living c2c12 cells are present, and the media is removed. Cells were rinsed with 4 mL sterile PBS, following which 0.5 mL trypsin was added to the culture dish and incubated at 37°C for 2 min. 2 mL of media was then added to the culture dish to wash off cells, following which the cells were collected, centrifuged, and a portion of the supernatant removed. The cells were plated onto the PDMS channels (channel side facing up and exposed to medium) and they were incubated in the channel in a 37°C incubator and viewed microscopically for attachment at 12 hour intervals for 72 hours. Unattached cells were removed by rinsing with sterile PBS. Cells attached preferentially to regions inside the channel because that is the only region coated with cell adhesive protein. Other regions were coated with BSA.

The cell-plated PDMS channels were then placed against a flat surface, channel side down, to provide a closed channel system with cells attached to the PDMS channel walls. The inlet reservoir itself can serve as the gravity pump reservoir or may also be attached to a horizontal fluid supply reservoir containing cell culture media. Culture of c2c12 cells under continuous, substantially constant flow is exhibited.

The separation, or "sorting" of motile from lesser-motile and/or non-motile particles has numerous applications, but heretofore has been technologically difficult. For example, in analysis of water supplies, it may be desirable to separate motile bacteria and other microorganisms, including fungi, algae, etc., from those which are non-motile. Identification and enumeration of the various species may thus be facilitated. Examples of motile organisms include flagellated and ciliated bacteria such as C. elegans, and other microorganisms, such as paramecia and motile plankton. Either the motile species enriched or motile species-depleted samples, or both, may be independently analyzed, cultured, etc.

An especially significant application is the sorting of sperm cells. For example, in the case of in vitro fertilization, if the donor's sperm count is low, and especially if contaminated with non-motile sperm, deformed sperm of lesser motility than the desired viable sperm, and other cells and seminal debris, the success rate is raised considerably when the motile sperm are used substantially for fertilization attempts. For example, avoidance of anueploid sperm or DNA fragmented sperm is particularly desirable. In many endeavors, it is desirable to be able to direct the gender of the offspring, for example when the birthing of milk cows is desired. In such cases, it would be advantageous to be able to sort the X- and Y- chromosome containing sperm based on their known motility differences.

Sperm cells from donors with oligozoospermia (low sperm count) have previously been concentrated and to some degree separated from cells and debris having different sizes and/or densities by centrifugation. However, this technique allows incorporation of non-gametes into the enriched sperm sample. These non-gametes, however few there are, release oxygen radicals which are detrimental to continued sperm viability. Moreover, centrifugation is a brute force technique which damages significant numbers of sperm, particularly at the mid-piece and tail regions.

So-called "swim up" techniques are also known, but isolation of the most viable sperm is challenging. S. Smith et al., FERTIL. STERIL. 1995, 63, 591-97. Thus, doctors frequently resort to hand sorting through dead sperm and debris to find sperm which are motile and of distinct morphology, a very time-consuming process.

Applications in biogenetics (biotechnology) also frequently require separation of particles based on their motility. In non-biological application, separation of working microrobots (which are motile) from non-working microrobots is a possible application.

Sorting of motile and non-motile or lesser-motile particles is accomplished by establishing a non-turbulent and preferably laminar flow stream

("sort stream") containing motile and non-motile or lesser-motile particles to be sorted, and contacting this sort stream with a second non-turbulent and preferably co-laminar media flow stream ("media stream"), providing an exit stream for at least a portion of a motile particle-enriched media flow stream, and an exit stream for a motile particle-depleted sort stream. The mobility of the motile particles allow them to enter the media stream along the interface between the media and sort streams, while non-motile or lesser-motile particles remain substantially within the sort stream. Apparatus suitable for use in the process provide for at least one sort stream inlet, at least one media stream inlet, at least one sort channel, at least one motile particle-depleted sort stream outlet, and at least one motile particle-enriched media stream outlet. The apparatus are preferably relatively small devices prepared by micromachining or polymer casting techniques, and preferably contain all necessary functionality integrated onto a single "chip."

FIGURE 1A illustrates a simple embodiment of a motile particle sorting device of the present invention;

FIGURE 2A depicts sorting of motile from non-motile particles in a sort channel of a device of Figure 1;

FIGURE 3A illustrates one embodiment of series connected sorting devices;

FIGURE 4A illustrates a further embodiment of a sorting device with multiple media inlets;

FIGURE 5A illustrates in schematic form a further embodiment of a sorting device in accordance with the present invention;

FIGURE 6A illustrates in schematic form a further embodiment of a sorting device in accordance with the present invention;

FIGURE 7A illustrates a device similar to that of Figure 1, in perspective; and

FIGURE 8A illustrates sorting efficiency of a device similar to that of Figure 1 in sorting sperm.

The description of the invention may be facilitated by reference to Figure 1 which represents one relatively simple embodiment of an apparatus which may be used for practicing the subject invention, and by reference to Figure 2 which illustrates sorting of motile from non-motile particles. Following these descriptions, additional details of the functioning of the apparatus, its geometry, the nature of fluids and fluid flow, etc., will be explained in greater specificity.

The device of Figure 1 is shown in schematic form in plan, i.e. as viewed from above. The device 1 embodiment consists of a motile particle sort stream inlet 2 (or motile particle supply reservoir serving as an inlet), a media stream inlet 3 (or media reservoir), a motile particle-depleted sort stream outlet (or reservoir) 4, and a motile particle-enriched stream outlet (or reservoir) 5. Between the inlets 2 and 3 and the outlets 4 and 5 is located a sort channel 6. Connecting the sort channel 6 to the respective inlets and outlets are sort stream inlet channel 7, media stream inlet channel 8, motile particle-depleted sort stream outlet channel 9, and motile particle-enriched media stream outlet channel 10. The width of the sort stream channel must be large enough to allow the particles of interest to pass through effectively without blockage, as is also true of both outlet streams. In general, the inlet streams and outlet streams will have a cross-sectional area less than the sort channel. The relative cross-sections will be dependent on the flow volume and flow rates of the various streams. The linear flow rates are preferably similar, although the relative flow rates are only limited by the occurrence of mixing between the sort stream and the media stream. Depending upon numerous factors such as the viscosities of the media and sort streams, the motility of the particles, and the presence or absence of particles or debris of different size than the particles desired to be sorted, the volume of the media stream may be less than, substantially

the same as, or greater than the volume of the sort stream over any section of the sort channel.

The bulk of the description which follows is described in relation to sorting of sperm, although the same principles apply to other motile and non-motile particle sources.

In operation, a supply of sperm is introduced into sort stream inlet 2 and caused to flow toward motile particle-depleted sort stream outlet 9, initially through channel 7, then through sort channel 6, and next to outlet channel 9. A media supply stream compatible (i.e. not destructive) of the sperm to be sorted is introduced into media stream inlet 3 and caused to flow through channel 8 into sort channel 6, through channel 10, and into motile particle-enriched media outlet 5. At the confluence of channels 7 and 8, a non-mixing, and preferably laminar flow is created, such that the sort stream and media stream flow in parallel through the sort channel. Non-motile (or lesser-motile) particles tend to remain in the sort stream, while motile particles move about randomly and enter the media stream. As a result of this random movement, the sort stream becomes depleted of motile sperm, while the media stream becomes increasingly enriched.

The invention may further be described broadly with reference to Figures 2a, 2b, and 2c, which illustrate pictorially the separation of motile from non-motile sperm and other non-motile particles in the sort channel of the device of Figure 1. In Figure 2a, the sort channel 6 is shown, beginning at the point of confluence of the sort stream 11 and the media stream 12. The sort stream 11 contains motile 13 and non-motile sperm 14 as well as other non-motile particles, here designated as "round cells" 15. Note that the size of the media stream in plan, and hence its volume, is considerably greater than the sort stream. Since sperm (and similarly, other motile particles) assume an essentially random distribution in the total liquid within a short period, a larger media stream volume will necessarily contain a larger fraction of total motile sperm 13.

In Figure 2b, the randomization of motile sperm between the two streams has begun, and continues until the desired degree of randomization has been achieved. This degree of randomization is preferably such that the concentration of motile sperm in the media phase per unit of volume is the same or greater than the concentration per unit volume in the sort stream. Note that the sort stream and media stream are maintained as separate streams, each preferably exhibiting laminar flow, and having a common boundary, or interface, 16. Greater concentration of motile particles in the media stream over the amount dictated by pure randomization may be achievable by employing a media stream in which the motile particles have increased mobility, i.e. by selecting a media stream less viscous than the sort stream, or by including additives which increase mobility of motile sperm relative to non-motile or poorly motile sperm.

In Figure 2c, the motile sperm-enriched media stream is harvested by diverting it to flow into the motile particle-enriched channel 10, while the now motile particle-depleted sort stream continues through channel 9 into outlet 4.

The diverting juncture 17 which separates the motile particle-depleted sort stream from the motile particle-enriched stream may be of any geometry which avoids substantial mixing of the streams at this point. The juncture 17 may be positioned, for example, to provide for substantially the same outlet channel configuration (i.e. height, width) of the sort stream inlet, at this point. To minimize contamination of the media stream by non-motile sperm, the juncture 17 may also be configured such that a small portion of the media stream is also directed to the motile particle-depleted sort stream outlet 9. In this case, a modest loss of motile sperm will occur, however, the probability that non-motile sperm may enter the motile sperm-enriched media stream will be lessened as a result.

The nature of the sort stream is not critical. The sort stream may be a biologically derived stream such as semen, or may be washed, diluted, may be treated with additives, stains or fluorescing dyes, viscosity modifiers, may be buffered, etc., so long as the treatment does not impair the viability of the desired exit stream (motile particle-depleted or motile particle-enriched). If separation but

not viability is the aim, for example with bacterial samples, the number of potential modifications of the sort stream are enlarged. The sort stream may also be a previously motile particle-depleted sort stream or motile particle-enriched media stream. For microrobotic devices, the sort stream liquid may be any which does not impair the functioning of the device, for example water, alcohols, ketones, glycols, esters, hydrocarbons, etc. With biological samples, water-based sort streams are ordinarily used.

The media stream may be selected with the same considerations in mind which are applied to selection or modification of the sort stream. In some cases, the sort stream may be water, but for biological systems, it is typical to employ streams which maintain or enhance biological activity, such as physiological saline, buffered saline, nutrient broths, and the like. In the case of human sperm, the preferred media is HEPES buffered human tubal fluid.

The nature of the media fluid and the sort fluid may be selected, if possible, to avoid interfacial mixing due to osmotic effects. This is the case, for example, when the base fluid (e.g. water) of both the sort and media streams have substantially the same amounts of soluble ingredients such as salts, acids, bases, buffers, dissolved organic material, and the like. The fluids may also be selected, when possible, to avoid interfacial mixing by diffusion. However, complete absence of any diffusion is an unlikely goal in this respect.

The relative fluid volumes may be selected with respect to the desired degree of incorporation of the motile particles within the media phase. For the highest degree of incorporation, the media volume should be large with respect to the sort fluid volume. However, proportionately smaller media volumes may also be used, particularly when sequential (serial) sorting is performed. Ratios of media fluid to sort fluid of from 1:1000 to 1000:1 are preferably used, more preferably 1:100 to 100:1, and most preferably within the range of 1:10 to 10:1. For typical applications, the ratio of media volume to sort fluid volume is within the range of 1:1 to 3:1. The media fluid volume is most preferably higher than the sort fluid volume.

The volumes referred to here are the volumes at a given cross-section of the sort channel. For example, a sort channel which is rectangular in shape having dimensions of 100 μm x 200 μm will have a "transverse volume" of 2 x 10^4 μm^2. This "transverse volume," actually a cross-sectional area, can be converted into true volume by multiplying by channel length or an increment thereof. Thus, the same rectangular channel previously described and having a transverse volume of 2 x 10^4 μm^2 will have an actual volume over a 100 μm length of 2 x 10^6 μm^3.

The linear flow rates of the sort fluid and media fluid are preferably substantially the same, *i.e.* within a range of flow rates of 1.5:1 to 1:1.5. If the linear flow rate of the media fluid is greater than that of the sort fluid, correspondingly less transverse volume of media fluid can be used for the same degree of motile particle incorporation. Flow is preferably concurrent, although counterconcurrent flow is also possible provided that interfacial mixing is not exacerbated beyond that which facilitates the desired degree of depletion/enrichment of the sort and media fluids.

The interface between the sort and media fluids is preferably a substantially non-mixing interface. By "non-mixing" is meant an absence of mixing which occurs due to excessive turbulence between the two fluids. For example, it is most desired that parallel, concurrent, laminar flow take place such that a substantially "static" appearing interface is obtained, as opposed to an interface which exhibits waves, currents, eddys, and the like. Turbulent flow generally results in partial to full mixing of the streams, rendering depletion/enrichment of motile particles less efficient or even completely impossible. The theoretically best resolution of motile particles occurs when a static-appearing interface or "streamline" is created where interfacial mixing occurs only due to diffusional and osmotic effects. However, it would not depart from the spirit of the invention to allow some turbulence along the interface. The turbulence is excessive when the desired degree of resolution cannot be obtained, even with multiple stages of devices. The turbulence, expressed as a Reynolds number, should in any case be less than 2000, more preferably less than 100, yet more preferably less than 10, and

most preferably 1 or less. High performance devices such as those illustrated by example herein, exhibit a Reynolds number of approximately 0.1.

The nature of the interface, i.e. its degree of turbulence, may be assessed by the degree of resolution. However, the turbulence may also be assessed in numerous additional ways. For example, in PDMS devices as described hereinafter, the optically transparent nature of the device allows the interface itself to be observed microscopically, for example by coloring one or both of the fluids and observing the interface by the change of color at the interface. By conventional optical techniques, the interface between media of differing refractive index are also easily observed. The degree of mixing of the sort and media streams may also be monitored by introducing a taggant, i.e. a radioactive soluble compound or non-motile particle, a visual or fluorescent dye, etc., into one stream but not the other. Appearance of the taggant in the outlet stream of the stream initially containing no taggant provides evidence of interfacial mixing, either of a turbulent kind, or by diffusion or osmosis. Some incorporation due to the latter two effects is expected, but is also expected to be quite minimal. An incorporation of 50% of the taggant into the non-tagged stream essentially constitutes complete mixing. Mixing of less than 20% of the taggant into the non-tagged stream, preferably less than 10%, more preferably less than 5%, and most preferably less than 1% is desired. So long as the Reynolds number is kept reasonably low, the degree of turbulence will be satisfactory. A flow which satisfies the above criteria is termed a "substantially non-turbulent flow" herein. It should be noted that concurrent flow streams exhibit much less turbulence, and hence interfacial mixing, than countercurrent flow streams.

Provided the fluid flow rate meets the non-turbulent requirements just described, the rate itself may vary widely. The walls of the sorting device also create the possibility for turbulence, since they are static with respect to the fluid flow. The effect of the walls will be most important when narrow channels are employed, and particularly at the walls which abut the narrower of the sort or media streams. Since the devices of interest are rather small and have rather small channels, linear flow rates of less than 10 cm/s, preferably less than 10 mm/s are

preferred. Flow rates of between 0.1 mm/s to 10 mm/s are particularly preferred. The low end of linear flow rate is determined by the mixing of non-motile particles from the sort stream into the media stream by Brownian motion. For example, at a flow rate of zero, with identical base fluid compositions (e.g. buffered saline), distribution of non-motile particles into the media phase would eventually be complete over time such that their concentrations become identical. The higher the flow rate, the less Brownian redistribution of non-motile particles will occur. The upper limit of the flow rate is reached when the interfacial flow becomes turbulent, as evidenced by a high degree of mixing.

Determining the relative flow volumes, relative flow rates, and absolute flow rates of any given stream can be routinely accomplished by one skilled in the art by simple calculations and/or measurements of resolution, for example by varying the respective rates and volumes and determining the relative enrichment and depletion of particles between the sort and media streams.

The geometry of the devices can vary. Sort channel length, for example, is generally a function of the rapidity at which motile particles randomize themselves between the two phases, and the flow rates. For example, at a given flow rate, motile particles which have limited motility will require a longer sort channel, while at a given sort channel length, less motile particles will require a slower rate of flow. Interfacial surface area also effects the geometry of the device. For example, flat rectangular sort channels with one fluid located parallel to and abutting a channel face of greater dimension, and with the other fluid adjacent, will exhibit faster randomization and thus require less sort channel length than the same channel when the first fluid is located parallel to and abutting a channel face of lesser dimension. In the latter case, the interfacial area is much reduced as compared to the former.

While it is theoretically possible to construct devices of macroscopic size, even of greater than 10 cm in length, for most purposes, the sort channel will be quite short, in almost all cases less than 2-5 cm, and for most devices, in the range of 100 μm to 1 cm. For sperm sorting, for example, a sort channel length of

5000 μm (5 mm) has proven quite satisfactory. In staged devices, shorter sort channel lengths may be desirable.

The cross-sectional geometry of the sort channel is not critical. Square, rectangular, ellipsoidal, circular, trapezoidal, triangular or other cross-sections may be used. For ease of manufacturing, non-undercut channels such as square, rectangular, triangular, trapezoidal, and half-round or half-elliptical sections are preferred. These shapes are preferred, for example, when neat casting or solution casting methods of construction are employed. In the case of construction by stereolithography techniques ("SLA"), more complex shapes can easily be fabricated. Complex shapes with undercut channels can also be provided by casting techniques when the device is cast in successive layers which are then attached together, for example by bonding. However, the channel width must be such that both the media stream and sort stream can both incorporate particles. For human sperm sorting, for example, a substantially rectangular channel with a height of 50 μm and a width of 500 μm has proven satisfactory. For a point of reference, human sperm have a head of about 2.5 μm in diameter and about 5 μm long, and are about 60 μm in overall length.

The cross-sectional areas and hence size of the supply channels and outlet channels are generally smaller than those of the sort channel. The minimum size of the sort stream inlet channel is dictated by the size of the particles which are present in the sort stream. Preferably, the sort stream channel provides a free channel from 3 to 10 times the size of the particles expected to be contained therein. The same considerations apply to the size of the media stream outlet channel, but not necessarily to the media stream inlet channel. Preferably, the sort stream inlet and outlet channels will have comparable sizes, although in some instances, as described earlier, it may be desirable that the outlet channel is larger than the inlet, thus incorporating a portion of the media stream into the sort stream. For sperm sorting, a rectangular sort stream inlet channel of 50 μm height, 100 μm width, and 5000 μm length has proven satisfactory.

The length of the various inlet and outlet channels is not critical. It is preferred that at least the inlet channels have some substantial length, to encourage formation of a laminar flow stream prior to the confluence of the sort and media stream channels. In general, more viscous fluids will not require as long a channel length as less viscous fluids. In some cases, the inlet channels may be completely dispensed with, *i.e.* the sort stream inlet (or reservoir) and/or media stream inlet (or reservoir) may feed directly into the sort channel. For most purposes, however, and to facilitate construction of sorting devices, it is preferable that inlet channels be employed. For the sperm sorting device described later, for example, inlet channel lengths of about 3 mm have proven satisfactory.

The junction 18 (Figure 1) of confluence of the sort and media streams is preferably configured to encourage a smooth joining of the fluid streams without excessive mixing. In general, therefore, the junction will be a relatively acute angle. The included angles between the sort stream inlet channel and the sort channel and between the media stream inlet channel and sort channel may be the same or different, *i.e.* the devices are not necessarily symmetrical. The same considerations apply to the junction 17 where the sort stream and media stream are separated, or "diverted" from each other. However, it is preferred that the sort stream inlet channel, sort channel, and sort stream outlet channel be substantially linear to provide as little disturbance of the non-motile particles in the sort stream as possible.

The material of construction of the sorting devices may be any suitable material, and the fabrication of the device may involve any fabrication process. For example, devices may be micromachined chemically by etching of glass, silica, silicon, metals, or by solution etching of polymers, etc. The devices may also be individually fabricated by known stereolithography techniques. The devices may be injection molded of moldable polymers, for example silicone rubber, thermoplastic polyurethane, polyethylene , polypropylene, polytetrafluoroethylene, polyvinyl chloride, polyvinylidene chloride, polyamide, polyester, and the like.

It is at present preferable to cast the sorting devices by supplying a negative "master" and casting a castable material over the master. Preferred castable materials are polymers, including epoxy resins, curable polyurethane elastomers, polymer solutions, *i.e.* solutions of acrylate polymers in methylene chloride or other solvents, and preferably, curable polyorganosiloxanes, most preferably for cost reasons, polyorganosiloxanes which predominately bear methyl groups, such as polydimethylsiloxanes ("PDMS"). Curable PDMS polymers are well known and available from many sources. Both addition curable and condensation-curable systems are available, as also are peroxide-cured systems. All these PDMS polymers have a small proportion of reactive groups which react to form crosslinks and/or cause chain extension during cure. Both one part (RTV-1) and two part (RTV-2) systems are available. Addition curable systems are preferred when biological particle viability is essential.

In many instances, transparent devices are desirable. Such devices may be made of glass or transparent polymers. PDMS polymers are well suited for transparent devices. A benefit of employing a polymer which is slightly elastomeric is the case of removal from the mold and the potential for providing undercut channels, which is generally not possible with hard, rigid materials. Methods of fabrication of microfluidic devices by casting of silicone polymers is well known. See, *e.g.* D.C. Duffy et al., "Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)," ANALYTICAL CHEMISTRY 70, 4974-4984 (1998). See also, J.R. Anderson et al., ANALYTICAL CHEMISTRY 72, 3158-64 (2000); and M.A. Unger et al., SCIENCE 288, 113-16 (2000).

The nature of the channel and reservoir walls of the devices may be selected in view of the application of the device and the fluids contemplated for use therein. For example, the walls may be hydrophobic or hydrophilic, or some portions of the device may be hydrophobic while other portions are hydrophilic. In addition, the walls may be treated or derivitized to modify their surfaces with biologically compatible or bioactive coatings, or to provide chemical functionality. For sperm sorting, coating the channels with bovine serum albumin (BSA) has

proven useful in improving liquid flow within the channels and to minimize non-specific adsorption of cells to channel walls.

Fluids may be supplied to the inlets or inlet channels of the device by any suitable method. Fluids may, for example, be supplied from syringes, from microtubing attached to or bonded to the inlet channels, etc. In preferred devices, the sort stream inlet and media stream inlet are in the form of "on-chip" reservoirs capable of holding and supplying the requisite amounts of liquids. These reservoirs may be filled by syringe, pipet, etc.

Fluid flow may be established by any suitable method. For example, external micropumps suitable for pumping small quantities of liquids are available. Micropumps may also be provided in the device itself, driven by thermal gradients, magnetic and/or electric fields, applied pressure, etc. All these devices are known to the skilled artisan. Integration of passively-driven pumping systems and microfluidic channels has been proposed by B.H. Weigl et al., PROCEEDINGS OF MICROTAS 2000, Enshede, Netherlands, pp. 299-302 (2000).

Preferably, however, fluid flow is established by a gravity flow pump, by capillary action, or by combinations of these methods. A simple gravity flow pump consists of a fluid reservoir either external or internal to the device, which contains fluid at a higher level (with respect to gravity) than the respective device outlet. Such gravity pumps have the deficiency that the hydrostatic head, and hence the flow rate, varies as the height of liquid in the reservoir drops. For many devices, a relatively constant and non-pulsing flow is desired.

To obtain constant flow, a gravity-driven pump as disclosed in published PCT application No. WO 03/008102 A1 (January 18, 2002), herein incorporated by reference, may be used. In such devices, a horizontal reservoir is used in which the fluid moves horizontally, being prevented from collapsing vertically in the reservoir by surface tension and capillary forces between the liquid and reservoir walls. Since the height of liquid remains constant, there is no variation in the hydrostatic head.

Flow may also be induced by capillary action. In such a case, fluid in the respective outlet channel or reservoir will exhibit greater capillary forces with respect to its channel or reservoir walls as compared to the capillary forces in the associated inlet channel or reservoir. This difference in capillary force may be brought about by several methods. For example, the walls of the outlet and inlet channels or reservoirs may have differing hydrophobicity or hydrophilicity. Alternatively, and preferably, the cross-sectional area of the outlet channel or reservoir is made smaller, thus exhibiting greater capillary force.

Most preferably, integrated, on-board reservoirs which serve as constant flow rate gravity-driven pumps and which also exhibit a difference in capillary forces between inlet and outlet are used. Flow in such devices may begin as soon as the devices are filled with liquid or when blocking valves or plugs are opened, or may be initially assisted by a pressure differential between the inlet and outlet, for example by applying suction briefly to the outlet.

Multiple devices may be connected in many ways to effect complex separations, to provide enhanced yield, to provide increased resolution (sorting efficiency) or any combination of these. In addition, multiple sort and/or media streams may be employed. When multiple sort or media streams are used, the sort streams may be the same or different, as may be the media streams.

For enhanced efficiency, for example, the motile particle-depleted sort stream outlet of a device such as that depicted in Figure 1 may be connected to the sort inlet of a second device, this second device also having a media supply. As a result of this sequential contact with two media streams, the sort stream will be further depleted of motile particles. The motile particle-enriched streams from both devices may be combined. Use of several sequential stages in this manner allows for virtually 100% recovery of motile particles. Preferably, when multiple devices are employed, they are fabricated on the same structure with integral connecting channels. One such device is shown schematically in Figure 3.

In Figure 3, the series configured two-stage motile particle sorter consists of a single sort fluid reservoir 20, connected to first sort channel 22 by sort stream channel 21. The first stage also consists of first media supply reservoir 23, media stream channel 24, motile particle-enriched first media channel 25, and motile particle-enriched first media reservoir 26. The motile particle-depleted sort outlet stream from the first stage flows through connecting passage 27 to serve as the sort stream inlet to the second stage sort channel 28. Second media reservoir 29 supplies media to the second stage sort channel through media inlet channel 30. Sort fluid further depleted of motile particles exits the device through channel 31 into motile particle-depleted sort stream reservoir 32, while a second stream of motile particle-enriched media fluid exits the sort channel through media outlet channel 33 and into reservoir 34. The two motile particle-enriched media reservoirs 26, 34 can be connected to a common exit channel or reservoir, optionally through valved passages, or may be emptied manually, *e.g.* using a syringe or pipet, and their contents combined, if desired.

Additional devices are shown in Figures 4, 5, and 6. In the device of Figure 4, two media supply reservoirs 41 supply media fluid to the device 40, motile particle-enriched media being collected in the two media outlet reservoirs 42. A single sort fluid reservoir supplies fluid containing motile and non-motile particles from sort fluid reservoir 43, and the motile particle-depleted sort fluid is collected in sort fluid outlet reservoir 45 after passing through sort channel 44. In this case, a central sort stream 46 is flanked on each side by media streams 47.

Figures 5 and 6 are schematics of multiple stage devices which rely on alternative connections of various flow paths to improve one or more aspects of the sorting process. In both Figures, double lines represent sort channels. The device of Figure 5 is capable of not only sorting motile from non-motile particles, but also into fractions of different motilities, and has three sort channels. The device of Figure 6 splits the outlet of a single sort channel into fractions, the furthest away from the sort stream containing proportionately more of the particles with highest motility. As can be seen, the present devices can be configured simply or with great complexity. Devices may also operate in parallel, series parallel, or

other modes. Parallel processing may be desired for sorting larger samples, or to measure sorting efficiency, etc., while comparing different media fluids. Such comparisons are more statistically accurate when measurements are made in a single device.

While much of the description herein refers to separation of motile from non-motile particles, the subject invention processes and devices are also suitable for separating motile particles of differing motility. The most motile particles will enter the media stream at a higher rate than particles of lesser motility. The residence time in the sort channel is preferably selected such that the most motile particles will assume a random or near random distribution in the total fluid. In contrast to separation of motile from completely non-motile particles, however, where additional sort channel length can be tolerated, and distribution of non-motile particles into the media stream is due substantially only to Brownian motion and to turbulence and like effects, when motile and lesser motile particles are separated, the lesser motile particles will also assume a random distribution given sufficient time. The sort channel length must be adjusted downward such that this cannot occur. The media stream will be enriched with both motile and lesser motile particles, but will be correspondingly more greatly enriched by the particles of greater motility. Multiple sequential processing of a first media stream (serving as the sort stream to a further stage) will cause higher resolution between the differently motile particles. Second and further sorting of the sort streams and their subsequent treatment in like fashion will increase the yield.

Example 1

A microfluidic sperm sorting device was prepared from Dow Corning SYLGARD® 184 curable silicone resin, using the soft lithography technique described by D.C. Duffy et al., cited previously. The curable PDMS was cast onto a master having the desired reservoir and channel features as protuberances. The cast PDMS sorting devices were plasma oxidized to seal the open channel side of the casting to a glass cover slide. Channels and reservoirs were coated with 1% bovine serum albumin fraction V from Sigma, dissolved in phosphate buffered saline

(PBS) from Invitrogen Corporation. The entire device was approximately 6 mm thick, exclusive of the cover slide, and somewhat larger than a U.S. penny coin. A perspective view of the device is shown in Figure 7.

In Figure 7, the PDMS casting is transparent, and only the reservoirs and channels are depicted. The cover slide would be bonded to the bottom plane of the device. The numerals are the same as those of Figure 1. The channels are rectangular in cross-section, with a channel height of 50 μm, while the reservoirs are roughly semi-circular. Both inlet reservoirs 2 and 3 are approximately 3 mm in height, while the outlet reservoirs are approximately 2 mm in height. The inlet and outlet channels 7, 8, 9, 10 are about 5000 μm long. The sperm inlet channel 7 and the motile depleted sperm outlet channel 9 have a width of 100 μm, while the media inlet channel 8 and outlet channel 10 have a width of about 300 μm. The sort channel 6 has a width of 500 μm and a length of 5000 μm.

Semen samples were obtained with institution Review Board approval from men undergoing infertility evaluation. Sorting tests were performed using washed semen samples. In the order listed, 60 μL of processing media was added to the media inlet reservoir, 50 μL of a washed semen sample to the sample inlet reservoir, and 2 μL of media to each of the outlet reservoirs. Sperm sorting yields were calculated taking these dilution factors into account. The numbers of motile sperm were determined by a Makler Counting Chamber (Sefi-Medical Instruments, Haifi, Israel). For visualization of membrane-compromised sperm, which generally corresponds to non-motile sperm, 3 μL of propidium iodide (Molecular Probes, www.probes.com, 60 mM dissolved in processing media) was added to sperm samples prior to sorting. A Texas Red filter set (577 nm excitation, 620 nm emission) was used to view red fluorescence from stained cells. An inverted microscope (NIKON TE 300, www.nikon-usa.com) with a CCD camera (Hamamatsu ORCA-100, www.hamamatsu.com) was used to capture images and record movies.

The sorting device uses a sorting system where non-motile sperm flow along their initial streamlines and exit one outlet whereas motile sperm can

deviate from their initial streamlines and exit through a different outlet. This sorting mechanism is related to the "filtering" mechanism used in an "H-filter" where rapidly diffusing small molecules exit through a different outlet from larger molecules and particles that diffuse more slowly. The difference between the two devices is that the sorting device of the present invention takes advantage of active movement of cells whereas an H-filter takes advantage of passive diffusion of particles. This type of sorting is possible because in small channels, multiple laminar streams can flow parallel to each other with no turbulent mixing at the interface between the streams. Typical Reynolds Numbers for the flow of sperm sample and media inside the sorting device were on the order of 0.1. Non-motile human sperm, approximately 60 μm in length, and non-motile particles on the same order of magnitude in size diffuse slowly ($D = 1.5x10^{-13}$ m^2/sec; 690 sec to diffuse 10 μm) and remained within their initial streamlines. In contrast, motile human sperm swim at velocities greater than 20 μm/sec at 20°C. This rapid mobility allows motile sperm, but not the non-motile sperm, to distribute themselves randomly within the width of a 500 μm channel within seconds. The sorting device was designed specifically to give sperm a residence time of 20 seconds in the main separation channel. A bifurcation placed at the end of this separation channel allows efficient collection of only the motile sperm that deviated from its initial inlet stream.

The sorting device described integrates all functions necessary for sperm sorting, for example, inlet/outlet ports, fluid reservoirs, pumps, power source, sort channel, etc., onto a simple chip design that is practical to manufacture and use. A key design feature of this embodiment is the set of four horizontally-oriented fluid reservoirs that also function as sample inlet/outlet ports and a fluid pumping system. The orientation, geometry, and size of these reservoirs are designed to balance gravitational forces and surface tension forces, and provide a pumping system that generates a steady flow rate over extended periods of time regardless of the volume of fluid in the reservoirs. This contrasts with conventional gravity-driven pumping systems whose flow rates decrease over time as the volume of fluid in the inlet reservoir decreases. The diameters of the reservoirs were selected to be small enough that surface tension prevents liquid from spilling out of

the horizontally-oriented reservoirs, but large enough to hold sufficient amounts of sample (tens to hundreds of microliters) and allow convenient sample introduction and recovery. This balance of forces allows the reservoirs to be arranged horizontally without the liquid inside spilling out. The horizontal reservoir arrangement, in turn, holds the height difference between the fluid in the inlet and outlet reservoirs the same (1.0 mm height difference between inlet and outlet reservoir ceilings) regardless of the volume of fluid present in the reservoirs and maintains a constant hydraulic pressure even as the amount of fluid in the reservoirs changes.

The passively-driven pumping system described here is unique in that it uses horizontally-oriented reservoirs to overcome the problem of traditional gravity-driven pumping, where the pressure decreases as the amount of liquid in the reservoir decreases. Furthermore, the structure of the pump is greatly simplified compared to other mechanical or non-mechanical pumping systems allowing easy manufacture and integration of the pump into a small, integrated device. Finally, the use of gravity and surface tension as the driving-force contributes to the overall small size of the sorting device by eliminating the need for power supplies such as batteries. Taking gravity, surface tension, and channel resistance into consideration, the sorting device was designed to give a steady flow rate of sperm with a residence time of approximately 20 seconds inside the main sort channel. More specifically, the device is designed so that the flow resistance of the fluid reservoirs is more than 10 times less than that of the microfluidic channels, and therefore negligible. Thus, the resistance of the channels, calculated to be 2.8×10^{12} kg/(sec/m^4), approximates the total resistance of the system. Since a bulk flow rate of 0.008 μL/sec is required to achieve the desired residence time of 20 seconds and the total resistance is 2.8×10^{12} kg/(sec/m^4), the net pressure drop required to drive the fluid is 23 N/m^2. To achieve this desired pressure drop, we designed the dimensions of the reservoirs such that capillary forces (3.0 mm diameter inlet reservoir vs. 2.0 mm diameter outlet reservoir) would be 13 N/m^2 and the pressure drop across the microfluidic channel of the sorting device due to hydrostatic forces (1.0 mm height difference) would be 9.8 N/m^2. For calculation of the capillary force, the contact angle was assumed to be 0° (the contact angle of water on BSA coated PDMS is very small),

the surface tension of the washed semen sample assumed to be approximately 0.040 N/m (less than that of water due to "impurities" such as proteins), and the viscosity of the washed semen sample to be similar to that of water. The observed bulk flow rate of 0.008 μL/sec for a dilute particle suspension in 1% BSA solution was approximately equal to that of the calculated flow rate. Actual sperm samples sometimes had lower flow rates due to larger apparent viscosity. Smaller flow rates for the sperm sample stream would result in slightly lower yields but does not affect the purity of the sperm recovered at the sorted sperm outlet.

Sperm sorting efficiencies of the sorting device were evaluated by three methods: (i) tracking the movement of motile sperm in the channel by phase contrast microscopy, (ii) tracking movement of propidium iodide (PI) stained cells in the channel by fluorescence microscopy, (iii) using a Makler Counting Chamber, a grid-based sperm counting device, to determine numbers of motile sperm and non-motile sperm in the inlet and outlet reservoirs (Figure 8). The sperm tracking experiments shows the process of how motile sperm can swim out of its initial streamline. PI stains membrane compromised cells such as dead cells, and thus allows the non-motile sperm to be highlighted and visualized with red fluorescence while the motile sperm remain unstained. The bar graphs in Figure 8 compare percentage of sperm that are motile before and after sorting. The unshaded bars represent the initial sperm sample, while the solid bars represent the motile particle-enriched media stream. The purity of motile sperm after sorting was nearly 100% regardless of motile sperm purity before sorting. The yields (39%, 42%, 43%), defined as the ratio of the number of motile sperm in the motile sperm outlet reservoir to the total number of motile sperm in the sperm sample inlet reservoir, were comparable to or greater than the recovery rates (0.8% to 50%) of sperm processed using conventional sorting methods such as direct swim-up, swim-up from a pellet of centrifuged sperm, or density gradient separation. It was also observed that sperm morphology, another important trait that correlates with successful pregnancies, also improved after sorting with the device (Strict Sperm Morphology: 9.5 ± 1.1% normal before sorting to 22.4 ± 3.3% normal after sorting). Kruger Strict sperm morphology is a set of criteria or standards whereby sperm must fit within specific measurements (head width and length, tail length, acrosome making

up a certain percentage of the sperm head) and lack abnormalities (*e.g.* pin head, round head, crimped tail).

As can be seen from the above, the motile particle sorting devices are small, easily manufactured, simple in operation, and highly efficient. In the claims which follow, the terms "a" and "an" mean "one or more than one" unless indicated otherwise.

While embodiments of the invention have been illustrated and described, it is not intended that these embodiments illustrate and describe all possible forms of the invention. Rather, the words used in the specification are words of description rather than limitation, and it is understood that various changes may be made without departing from the spirit and scope of the invention.

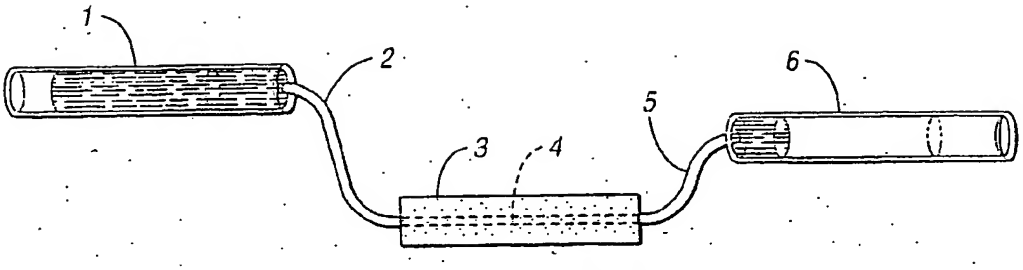


Fig. 1

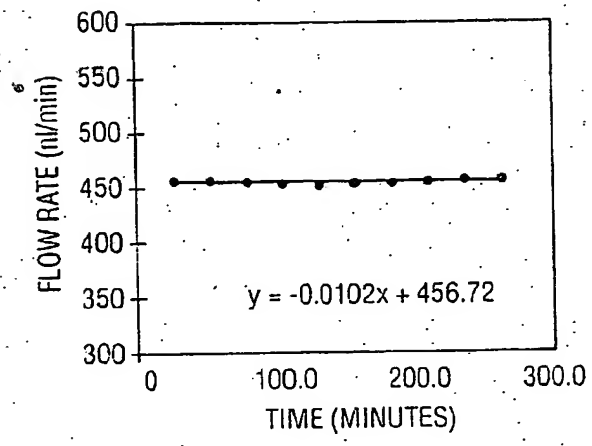


Fig. 2

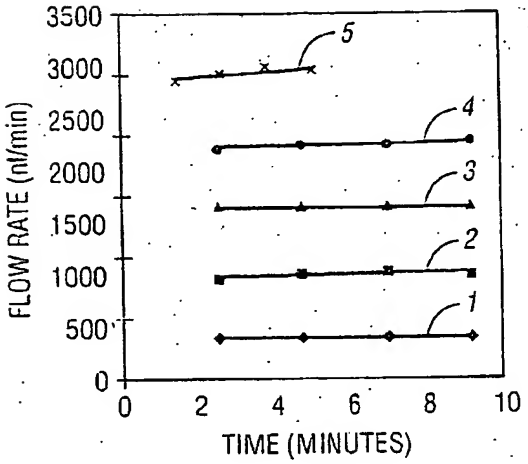


Fig. 3

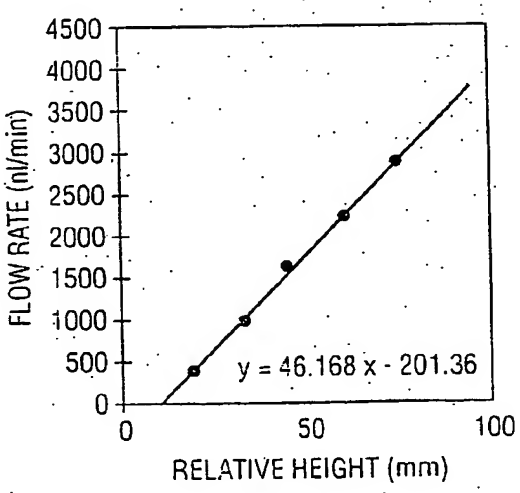


Fig. 4

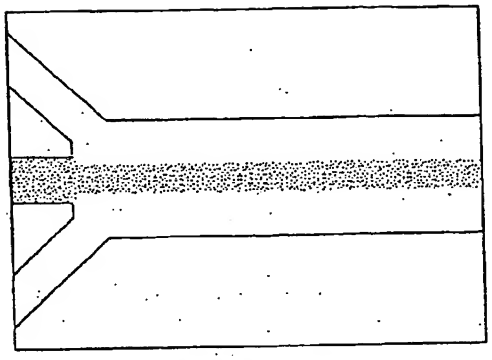


Fig. 5

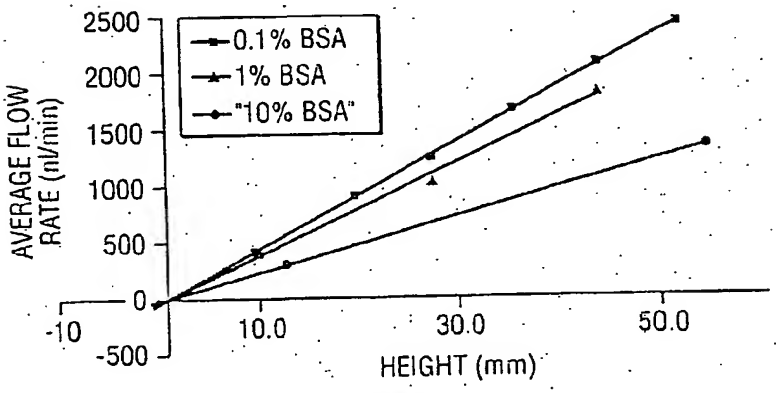


Fig. 6

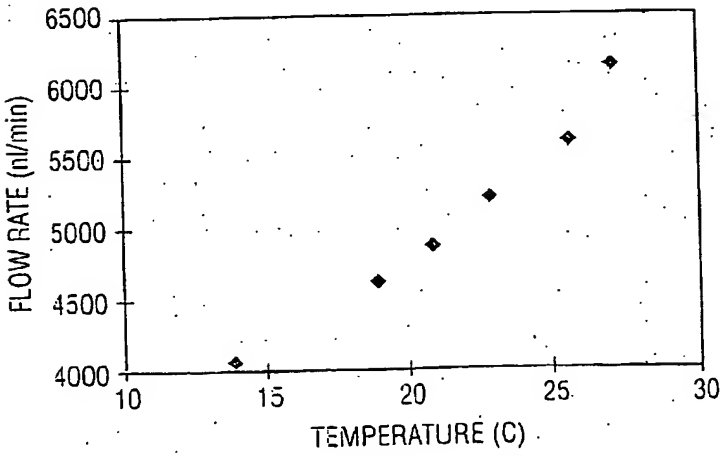


Fig. 7

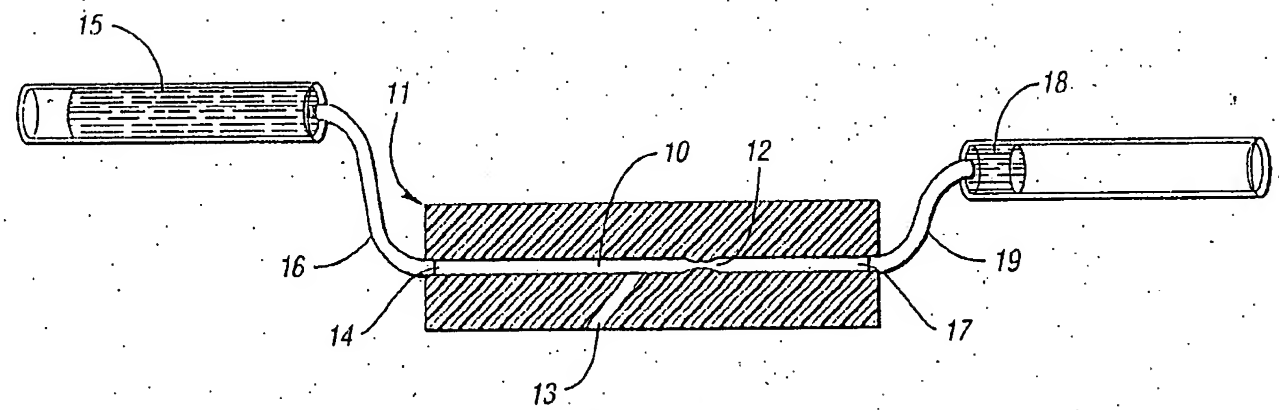


Fig. 8

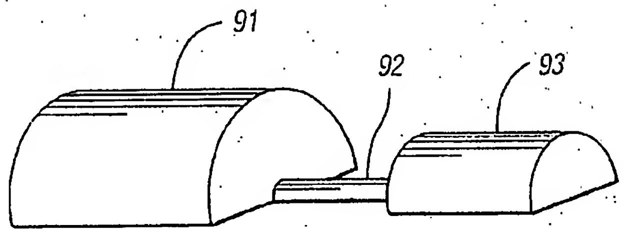


Fig. 9

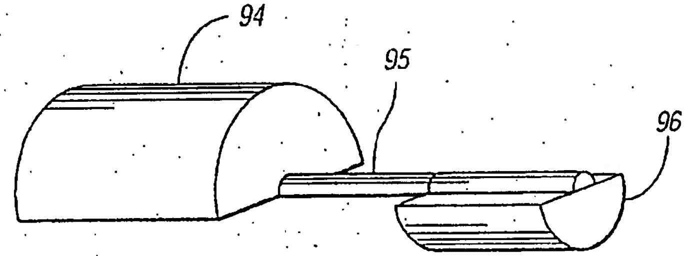


Fig. 10

3546

Integration of Egg Insemination with MISS-Isolated Sperm (12-17-02)

Sperm Bound to Zona Pellucida - Fertilized

2546

(12-17-02)

Integration of MSS Sperm Isolation and Microfluidic Channel Egg Insemination

Fig 2

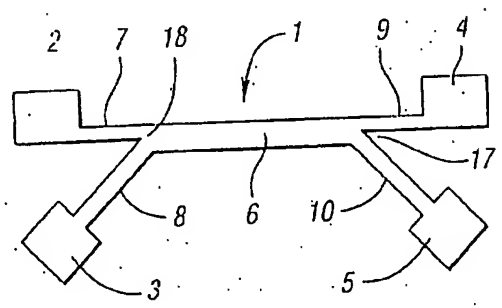


Fig. 1A

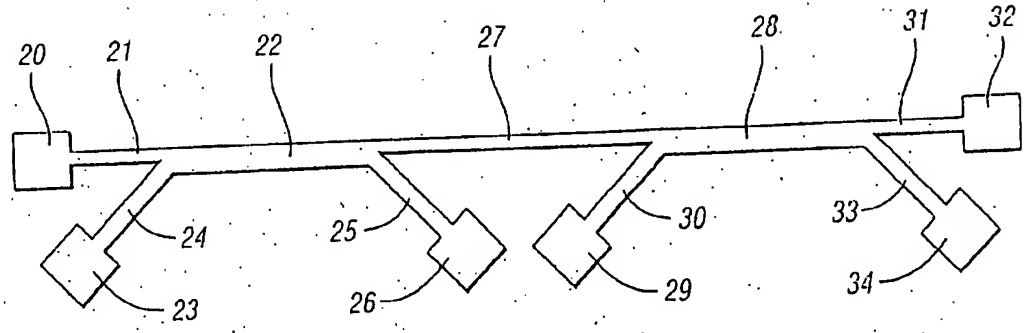


Fig. 3A

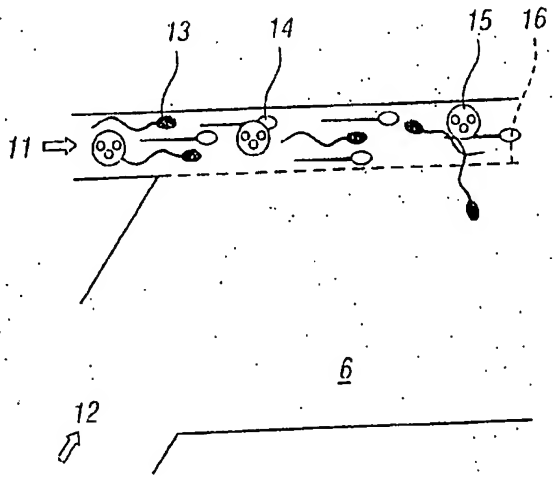


Fig. 2aA

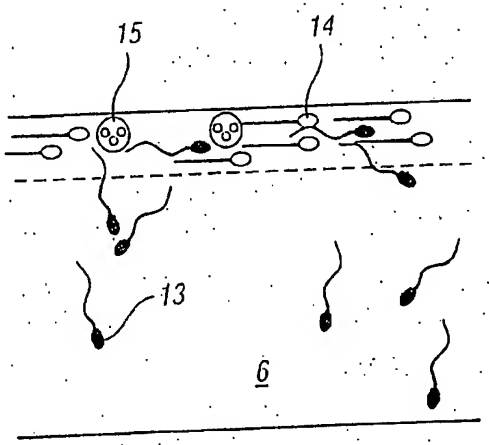


Fig. 2bA

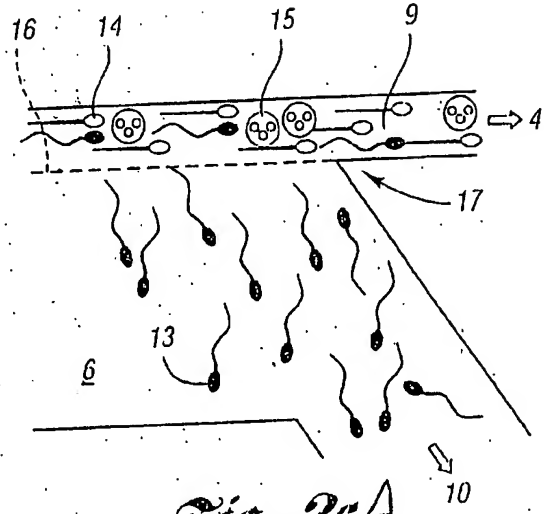


Fig. 2cA

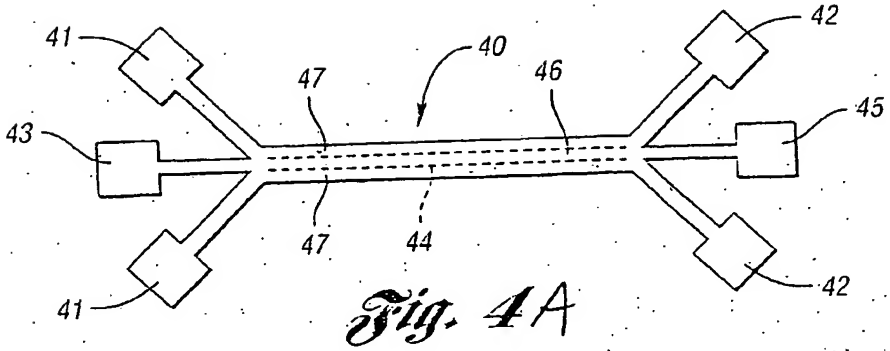


Fig. 4A

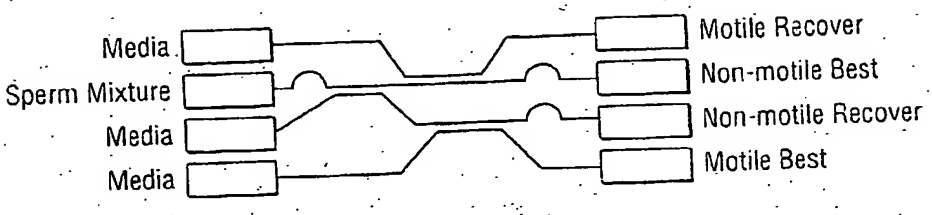


Fig. 5A

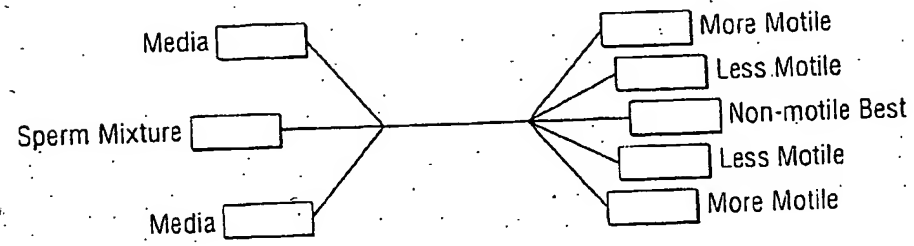


Fig. 6A

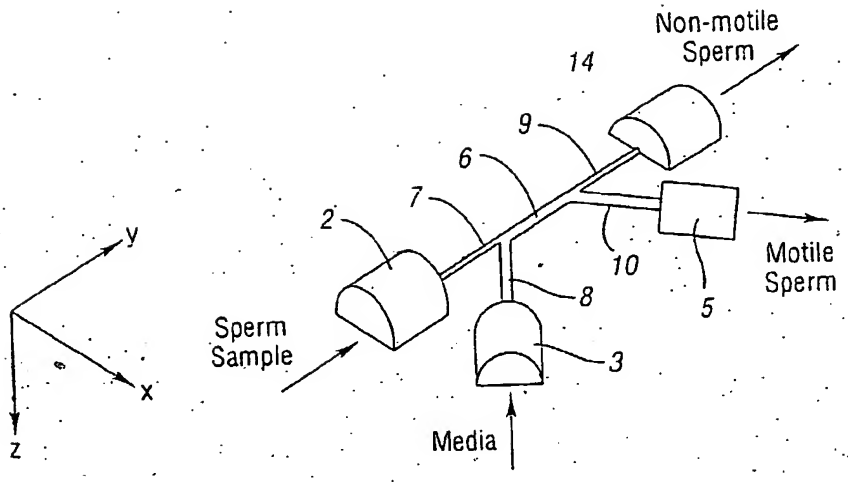

Non-motile
Sperm
14
9
6
7
2
10
8
5
Motile
Sperm
y
x
z
Sperm
Sample
3
Media

Fig. 7A

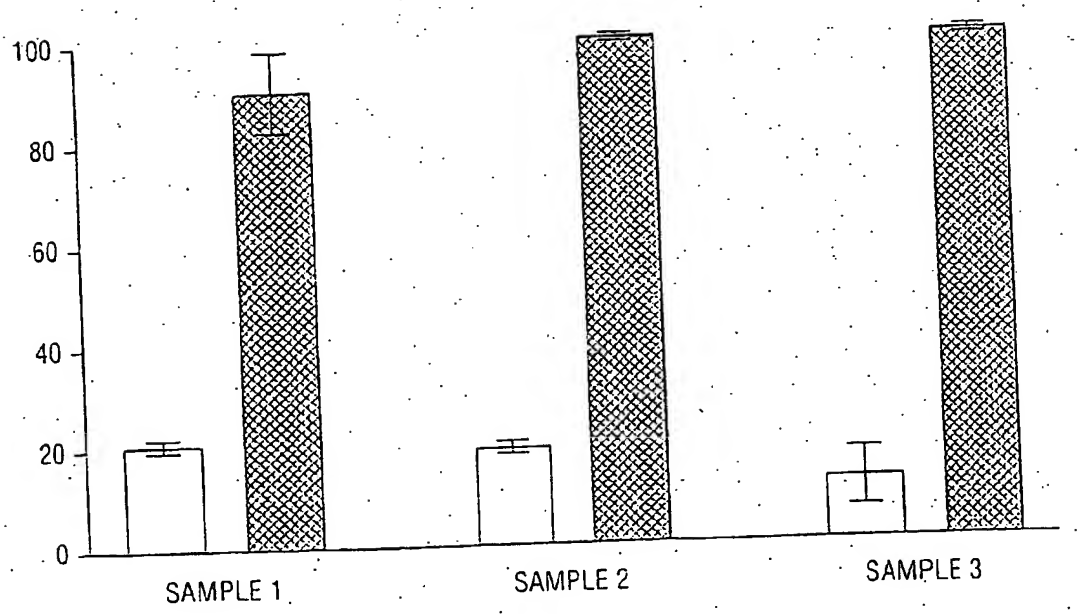

100
80
60
40
20
0
SAMPLE 1
SAMPLE 2
SAMPLE 3

Fig. 8A